*MOSSBAUER SPECTROSCOPY OF THE IRON-SULFUR PROTEINS**

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Abstract.—The Mössbauer spectra of ^{57}Fe in two plant ferredoxins (from spinach and *Euglena*) and in xanthine oxidase have been measured at a series of temperatures and magnetic fields, and are found to be similar in all three proteins. In the oxidized state the iron is nonmagnetic. In the reduced state, the iron nuclei show magnetic hyperfine interaction with an electron spin S of $^1/_2$, producing an effective field of about 180 kG at the nuclei.

The iron-sulfur (or nonheme iron) proteins were first classified and characterized by their similar and unusual electron paramagnetic resonance (EPR) spectra with g-values centered around 1.94 in the reduced state,[1, 2] although their molecular structure and the ligand environment of the iron were (and still are) unknown. Similarities were also evident in their optical spectra.[3] Their Mössbauer spectra,[4–10] however, have not so far always shown obvious similarities. The object of this paper is to compare data which we have previously reported on three of these proteins, xanthine oxidase,[4] spinach ferredoxin,[7] and *Euglena* ferredoxin,[8] and which we recognize to be similar. Further measurements are reported on spinach ferredoxin and xanthine oxidase which confirm that the iron atoms are in a similar state in all these proteins.

Mössbauer effect measurements have been made on ^{57}Fe in xanthine oxidase,[4] in ferredoxin from *Clostridium pasteurianum*,[5] *Chromatium*,[6] spinach,[6, 7] and *Euglena*,[8] in putidaredoxin,[9] and in *Azotobacter*.[10] These gave no EPR signal in the oxidized state and the $g \sim 1.94$ signal in the reduced state. Their Mössbauer spectra in the oxidized state all consisted of a doublet, as shown in Figure 1, but in the reduced state marked differences have been reported. Xanthine oxidase,[4] *Euglena* ferredoxin,[8] and putidaredoxin[9] showed magnetic hyperfine splitting in their spectra at low temperatures where the electron-spin relaxation times were long. Spinach ferredoxin was reported to show either no hyperfine splitting[6] or, when a small magnetic field was applied,[7] splitting for only about half the iron atoms. In the remaining proteins, measurements on the reduced state were not reported.

We shall first consider reduced *Euglena* ferredoxin[8] which in many ways seems to be the best-understood system so far. The *Euglena* was grown with enriched ^{57}Fe so that the intensity of the absorption was high, and yet damage to the molecule by incorporation of the isotope could not occur as it might when, for example, $^{57}Fe^{2+}$ is exchanged for the iron in the ferredoxin after it has been separated from the organism. The predominant features of the Mössbauer spectra at 4.2°K could be explained in terms of an almost isotropic hyperfine interaction $\mathcal{H} = AS \cdot I$, where I is the nuclear spin and the electron spin S is equal to $^1/_2$. This gives a prominent line at about 3 mm/sec in the zero-field spectrum and other

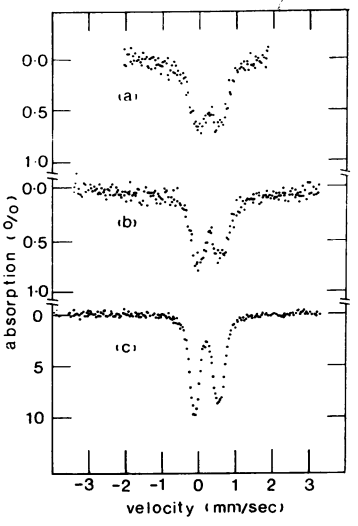

FIG. 1.—^{57}Fe Mössbauer spectra at 77°K of oxidized (*a*) xanthine oxidase, (*b*) spinach ferredoxin, and (*c*) *Euglena* ferredoxin. The zero of velocity is the center of the spectrum of iron at room temperature. Samples were prepared as described in refs. 4, 7, and 8, respectively.

broader and fainter lines at velocities down to −6 mm/sec. When an external magnetic field H greater than the hyperfine field at the electrons $H_e = A/2\,g\beta$ was applied, the spectrum showed a Zeeman splitting corresponding to an effective magnetic field $|H_n| = 180$ kG at the iron nuclei; the spectrum then became more symmetrical and had sharper lines. With the field perpendicular to the direction of the γ rays, six transitions were observed with relative intensities approximating to the theoretical values of 3:4:1:1:4:3; with the field parallel to the γ rays, two of these lines had zero intensity, and the relative intensities were observed to be in the predicted ratio of 6:0:2:2:0:6. It is important to note for our later discussion that the strong line at 3 mm/sec is not shifted in energy by the application of a small field, and that it becomes most intense when the field is applied parallel to the γ rays.

In spinach ferredoxin, previous work[6, 7] has shown that there was also a line at 3 mm/sec in the reduced state, and this was interpreted as arising from the higher energy transition in a quadrupole split spectrum from high spin Fe^{2+} ions. The lower energy transition was assumed to be near 0 mm/sec and was not resolved owing to the presence of two strong lines in the center of the spectrum, which were ascribed to iron in the unreduced enzyme. An alternative possibility that is now apparent is that the 3 mm/sec line could have a similar origin to that observed at the same velocity in reduced *Euglena* ferredoxin, but that the accompanying lines in the spectrum were obscured by the two strong central lines. Indeed, a close look at our earlier spectrum (Fig. 1*c* of ref. 7) shows traces of absorption at −6 and −3 mm/sec, but they were too weak to be clearly distinguished from noise and their significance at that time was not properly recognized. Moreover, the spectrum in a 5-kG magnetic field perpendicular to the γ rays (Fig. 2*a* and *b*, of ref. 7) showed very clearly a magnetic hyperfine pattern with an $|H_n|$ of about 150 kG, which was similar to that of *Euglena* ferredoxin, except that there were the additional strong lines in the center already mentioned. At the time of these measurements, however, the magnetic spectrum was assumed to have arisen from the

splitting of a high spin Fe^{2+} spectrum, although it was noted that this interpretation did not fit well with all the observed features.

The first clear observation of hyperfine interactions in an iron-sulfur protein was on reduced xanthine oxidase,[4] which was also the first such protein to be studied in the reduced state by Mössbauer spectroscopy. However, measurements were not made with a magnetic field applied parallel to the γ rays, which we have already noted as the best geometry for observing the magnetic hyperfine spectrum, since the lines are then fewer in number and therefore deeper. The optimization of the experimental conditions is important for our samples of spinach ferredoxin and xanthine oxidase since they were not enriched in ^{57}Fe and Mössbauer absorption was weak.

In order to confirm that the three proteins behaved similarly, we have measured the Mössbauer spectra of reduced spinach ferredoxin and xanthine oxidase at 4.2°K in the presence of a small (100 G) field applied along the direction of the γ rays. These spectra are shown in Figure 2 together with that of *Euglena* ferredoxin. It is clear that the three iron-sulfur proteins give lines at approximately the same energies, although the intensities are very different because of the varying amounts of ^{57}Fe in the samples studied. The appearance of hyperfine splitting with $|H_n| = 180$ kG shows that the iron has a similar electronic state in all three proteins, as has already been surmised from their similar EPR and optical spectra.

Figure 3 shows the spectra of reduced spinach ferredoxin in zero magnetic field at temperatures of 195, 77, and 4.2°K. These spectra are similar to those of ^{57}Fe-enriched *Euglena* ferredoxin at the same temperatures (cf. Fig. 2 of ref. 8), again

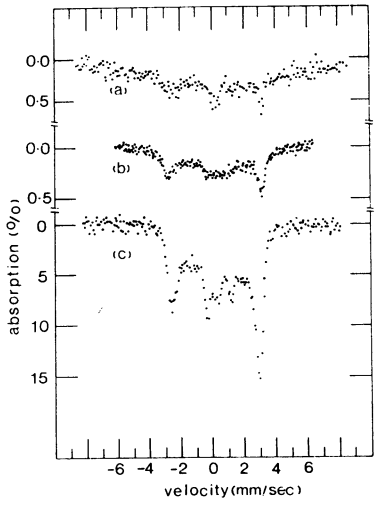

FIG. 2.—Mössbauer spectra at 4.2°K in a field of 100 G parallel to the γ rays of reduced (*a*) xanthine oxidase, (*b*) spinach ferredoxin, and (*c*) *Euglena* ferredoxin. All samples were reduced with added dithionite.

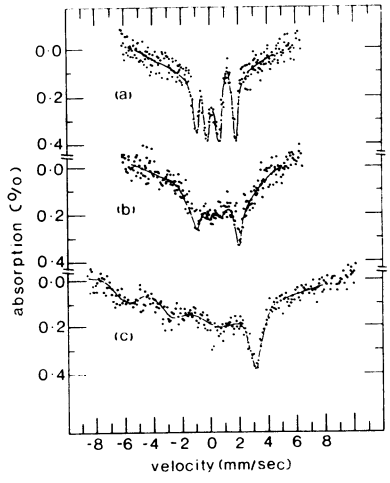

FIG. 3.—Mössbauer spectra of reduced spinach ferredoxin at (*a*) 195°K, (*b*) 77°K, and (*c*) 4.2°K.

showing that the iron is in identical states in the ferredoxins from the two sources. In the spectra of reduced spinach ferredoxin shown in Figures 2*b* and 3, the two central lines near zero velocity which were seen in the earlier published spectra[6, 7] were not observed. They were also absent or greatly decreased in the spectra of *Euglena* ferredoxin. These central lines, seen previously in reduced spinach ferredoxin, were probably due to the denaturation of the protein, which evidently has been diminished in the more careful preparation of the present specimen of reduced spinach ferredoxin. The precise conditions for avoiding denaturation have not yet been established and the occurrence of these lines has not yet been entirely avoided (Fig. 2*b*).

In conclusion, the Mössbauer spectra show that all the iron atoms in the proteins (two in the ferredoxins studied here and eight in the xanthine oxidase molecule) are equivalent in the reduced state, since they give only one spectrum. For these ferredoxins, the reduction is known to be a one-electron process (from visible and EPR spectroscopy measurements),[11–14] so that the unpaired electron is shared equally between the two iron atoms in the molecule. Garbett *et al.*[3] have suggested that xanthine oxidase contains four identical pairs of iron atoms while spinach contains only one pair (see also ref. 15). This interpretation agrees with our results.

The electronic state of the iron atoms in the oxidized and reduced proteins is still a matter for speculation. A model will have to explain that the oxidized state is diamagnetic ($S = 0$) and that, on reduction, the transfer of one electron per pair of iron atoms gives rise to a state with $S = \frac{1}{2}$. A number of models have been put forward by various workers. On the basis of current information, we suggest that the two most likely models (refs. 15–17) are:

$$\underset{\text{low-spin}}{\overset{d^5}{Fe^{3+}}\uparrow} \quad \underset{\text{low-spin}}{\overset{d^5}{Fe^{3+}}\downarrow} \quad \xrightarrow{e^-} \quad \underset{\text{low-spin}}{\overset{d^6}{Fe^{2+}}} \quad \underset{\text{low-spin}}{\overset{d^5}{Fe^{3+}}\uparrow} \qquad \begin{matrix} (S = 0) & (S = {}^1/_2) \end{matrix} \tag{i}$$

where the spins in the oxidized state are strongly antiferromagnetically coupled to give the $S = 0$ state, and

$$\underset{\text{low-spin}}{\overset{d^6}{Fe^{2+}}} \quad \underset{\text{low-spin}}{\overset{d^6}{Fe^{2+}}} \quad \xrightarrow{e^-} \quad \underset{\text{low-spin}}{\overset{d^7}{Fe^{+}}\uparrow} \quad \underset{\text{low-spin}}{\overset{d^6}{Fe^{2+}}} \qquad \begin{matrix} (S = 0) & (S = {}^1/_2) \end{matrix} \tag{ii}$$

It has been pointed out[16] that, with larger electron delocalization on to sulfur, this reduced state is formally equivalent to:

$$\underset{\text{low-spin}}{\overset{d^5}{Fe^{3+}}\uparrow} \quad (2\ RS^-) \quad \underset{\text{low-spin}}{\overset{d^6}{Fe^{2+}}} \qquad (S = {}^1/_2)$$

In some ways (ii) is the simpler possibility as it does not require antiferromagnetic coupling between the irons in the oxidized state. We anticipate that further experiments using the Mössbauer technique coupled with other physical measurements, e.g., EPR, will permit us to distinguish between these two possibilities.

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